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Award Number: DAMD17-02-1-0556

TITLE: A Novel Method to Screen for Dominant Negative ATM Mutations in Familial Breast Cancer

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REPORT DATE: April 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2004	Annual (1 Apr 2003 - 31 Mar 2004)

4. TITLE AND SUBTITLE
A Novel Method to Screen for Dominant Negative ATM Mutations in Familial Breast Cancer

5. FUNDING NUMBERS
DAMD17-02-1-0556

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The aim of this proposal is to identify families carrying potentially pathogenic *ATM* mutations by assaying for ATM kinase activity in cell lines derived from individuals with multiple cases of breast cancer in their family but no pathogenic *BRCA1* or *BRCA2* mutation ('BRCAx' families). If pathogenic mutations in *ATM* are found in these families it will substantiate the role of *ATM* in breast cancer susceptibility, allow us to characterise the functional effects of those mutations, and provide clinically valuable information for the families involved. We have analysed 252 cell lines established from index cases from non-BRCA1/2 breast cancer families for ATM expression and activity. 12/252 (4.7%) cell lines showed markedly reduced ATM kinase activity, despite normal level of expression of ATM protein. DNA from 6 index cases with aberrant ATM kinase activity has been screened D-HPLC and pathogenic *ATM* mutation have been identified in 2 index cases. Microarrays have been performed to determine differences in gene expression between heterozygotes (with or without breast cancer) or homozygotes for the T7271G mutation against each other and wild type control pools. These data have suggested candidate genes that are altered in their expression in T7271G *ATM* mutation carriers. The data must now be verified and can then be challenged with other carriers of ATM T7271G to see if it can be used to develop an alternate method for high-throughput screening of ATM mutations

14. SUBJECT TERMS
Familial breast cancer, ATM mutations, ATM activity, cDNA microarray

15. NUMBER OF PAGES
18

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

The *ATM* gene is mutated in the autosomal recessive disorder, ataxia telangiectasia (A-T), which is characterised by cancer predisposition, cerebellar ataxia and immunodeficiency. One of the most controversial topic in breast cancer genetics is whether mutations in the *ATM* gene predispose women to breast cancer. Studies of A-T families appear to have an elevated frequency of breast cancer in females, particularly in obligate heterozygotes whose risk may be increased as much as 7-fold. By contrast, most studies of sporadic breast cancer have not found an increased frequency of germline *ATM* mutations compared with controls, and linkage analysis of markers close to *ATM* in multiple-case families has provided no evidence that the *ATM* gene predisposes women to breast cancer. Nevertheless, two recurrent *ATM* mutations, T7271G and IVS10-6T->G, were recently reported to be associated with breast cancer (Stankovic *et al.*, 1998; Broeks *et al.*, 2000). We analysed these two pathogenic mutation in ATM in female-breast cancer only, non-*BRCA1/2* families in the Australian based Kathleen Cunningham Foundation Consortium for Research into Familial Breast Cancer (kConFab) and observed that 3% of the families carried one of the two mutations in *ATM* analysed (Chenevix-trench et al, 2002). We have shown that both mutations act as dominant negatives in that ATM kinase activity was markedly reduced in the heterozygous carriers of these mutations. These observations suggest that a proportion of hereditary breast cancer may be due to *ATM* mutations, and that the increased breast cancer risk may be restricted to a subset of *ATM* mutations.

The aim of this proposal is to identify families carrying potentially pathogenic *ATM* mutations by assaying for ATM kinase activity in cell lines derived from individuals with multiple cases of breast cancer in their family but no pathogenic BRCA1 or BRCA2 mutation ('BRCAx' families). In addition, we aim to identify target genes dysregulated by *ATM* mutations, and ideally to develop a novel method of high throughput screening for *ATM* mutations.

Our hypotheses were that:

a) Impaired activation of ATM kinase in lymphoblastoid cell lines (LCLs) from index cases of multiple case non-BRCA1/2 breast cancer families will provide a high-throughput screening method for identifying families carrying dominant negative mutations in *ATM*
b) Microarray analysis of LCLs from heterozygotes with dominant negative *ATM* mutations, and of stable transfectants generated with mutant *ATM* constructs, will identify a unique set of target genes dysregulated as a consequence of these *ATM* mutations. The information gained can be used to develop an alternate method for high-throughput screening and may also provide candidates for the genes involved in the genesis of ATM-related breast tumours.

This project will establish whether impaired activation of ATM kinase and/or microarray analysis can be used as powerful tool (s) to identify families carrying dominant negative

mutations in *ATM*. This will have important clinical ramifications for the families involved in terms of pre-symptomatic diagnosis, surveillance and risk management.

Body

Task 1 - Assay for ATM kinase activation in LCLs from the youngest affected person from 260 high-risk breast cancer families without a pathogenic mutation in BRCA1 and BRCA2 (months 1-6).

At our request, kConFab have established EBV-transformed cell lines from index cases (the youngest affected individual from each family) from 252 high-risk breast cancer families without a pathogenic mutation in BRCA1 or BRCA2. ATM expression was determined by western blotting with anti-ATM antibody and the activation of ATM was measured *in vivo* using anti-phospho-specific antibodies against the ATM phosphorylation site in p53 (phosphoSer15). Previous work from the laboratory and others have shown that ATM is required only for the immediate and rapid phosphorylation of its targets after ionizing radiation since they are still phosphorylated in ATM-null cells, albeit with delayed kinetics (Khanna *et al*, 1998, Gatei *et al*, 2001). Therefore, the activation of ATM was assayed within 30 mins of exposure to ionizing radiation (6 Gy). 42 out of the 252 cell lines tested showed reduced phosphorylation of p53 on Ser15, although the level of expression of ATM was quite comparable to controls. These 42 lines were retested again for Ser15-p53 phosphorylation and only 12 of these showed consistently reduced p53 phosphorylation. We have also noticed that p53 phosphorylation does not allow the assessment of ATM heterozygosity with 100% accuracy as it is influenced by cell culture condition and cell density etc. The 12 cell lines with consistently reduced p53 phosphorylation in 3 independent experiments also showed reduced phosphorylation of another ATM target CHK1 on Ser 317.

Task 2 - Microarray expression profiling with LCLs from 2 families each with members with the T7271G mutation, with and without prior exposure to ionising radiation

Initial pilot microarray experiments compared the following samples in pair-wise experiments:
1) wild type Vs T7271G homozygote lymphocytes after exposure to 3 Gy radiation and incubation for 0, 2, 6 and 12hrs.
2) wild type Vs T7271G heterozygote lymphocytes after exposure to 3Gy radiation and incubation for 0, 2 6 and 12hrs.

These experiments established that there are differences in the transcriptional response of wildtype Vs ATM mutant lymphocytes. Functional annotation of these differentially expressed genes revealed a large number of signal transduction molecules as well as genes of no known function.

Completion of microarray experiments and data analysis

We have now carried out extensive microarray experiments on 2 families with the T7271G mutation, Family 1 (Stankovic et al., 1998) and Family 2 (kConFab). RNA was extracted from LCLs from both families containing members with the T7271G mutation with and without prior exposure to ionising radiation. The RNA was pooled into 6 pools on the basis of phenotype:

1) unrelated control individuals with wild type ATM
2) heterozygous carriers of the T7271G mutation from family 1
3) homozygous for the T7271G mutation from family 1
4) control individuals with wild type ATM - family 2 (kConFab)
5) heterozygous carriers of the T7271G mutation who are not affected with breast cancer - family 2 (kConFab)
6) heterozygous carriers of the T7271G mutation who are affected with breast cancer - family 2 (kConFab)

Differences in gene expression between these pools were assessed using a total of 46 Compugen 19,000 gene long-oligo microarrays. Each hybridisation was carried out in triplicate and a dye swap was included. To understand gene changes that may be present between the ATM pools and to determine whether microarray analysis can be used to identify families carrying dominant negative mutations in ATM the experiment was divided into 4 sections (Fig. 2):

1. Comparison of different ATM genotypes - heterozygous, homozygous and wild type
2. Comparison of profiles for heterozygotes with breast cancer Vs heterozygotes without cancer, relative to wild type
3. Comparison of all pools via a common wild type ATM reference
4. Pair-wise comparisons of each pool before and after exposure to IR

Data extraction and image analysis of all microarrays was carried out using ImaGene5.0 (BioDiscovery Inc), and data warehousing tools established in house have been used to manage and store data (BASE: Saal *et al.*, 2002; http://microarray.imb.uq.edu.au/BASE). Subsequent analysis and data mining were performed with BASE and GeneSpring5.5 (Silicon Genetics Inc). Quality assessment of the microarrays was carried out using "arrayplots" tool in BASE and arrays that failed quality checking were repeated. To allow comparison of the microarrays several transformation steps were carried out on each array. Initially print tip Loess normalisation was carried out within each array, whereby a Loess curve fitted to the Log-intensity versus Log ratio plot was used to adjust the control value for each measurement. Scaling between each microarray was then performed. The normalisation steps for all 46 microarrays can be visualised as box plots (Fig. 3).

In the case of all direct comparisons, differential expression was defined using a robust statistical method rather than simple fold change (ie. Change more than 2 fold}. All genes were ranked using the B statistic (Sandiot and Speed's 2001) method where both fold change and variance of signals in replicates is used to determine the likelihood that genes are truly differentially expressed. Data from B-statistics can be visualised with a volcano plot whereby the probability (B-score) is plotted against the Log fold change for each gene, thus significance and magnitude of each gene between conditions can be visualised

(Fig. 4). A threshold in the B statistic of 0.0 was adopted as genes with a B score>0 have a >50% probability of being truly expressed. This analysis was executed using the Bioconductor package that has been implemented as a plug in tool in BASE. The ranked B-scores for all genes in each experiment can be obtained from BASE http://kidney.scgap.org/BASE.

Comparison of different ATM genotypes - heterozygous, homozygous and wild type

Family 1 was used to examine differential gene expression between ATM genotypes (heterozygous and homozygous) relative to an unrelated wild type pool (Fig. 2A). We found 1560 and 774 genes that were differentially expressed in heterozygotes and homozygotes compared to wild type, representing 8% and 4% of genes on the arrays respectively. A comparison of these differentially expressed genes showed 23 genes that were down regulated in both heterozygotes and homozygotes compared to wild type. These genes were linked to Gene Ontology functional annotations and over-representation of protein kinase cascade and NF-kappaB cascade genes was seen with p-values of 0.000174 and 0.000386, respectively. A further 206 genes were overexpressed in both heterozygote and homozygote pools in comparison to the unrelated wild type pool. There were also gene changes that differed between the heterozygous and homozygous genotype within Family 1, as in a direct comparison of these pools a total of 351 genes (2%) were significantly differentially expressed.

Comparison of profiles for heterozygotes with breast cancer Vs those with no disease, relative to wild type

Carriers of the ATM T7271G mutation in Family 2 were used to analyse changes in gene expression that may be associated with the genesis of ATM-related breast tumours (Fig. 2B). In a direct comparison of heterozygotes with breast cancer and those without, 100 genes differed in their expression. 65 of these were down regulated in the heterozygotes with breast cancer and were linked to Gene Ontology similarity over representation of genes involved in developmental processes with a p-value of 0.0296.
The 2 separate pools of ATM T7271G heterozygotes, those with cancer and those without cancer, were also compared relative to an internal family ATM wild type pool. 35 genes were found to vary significantly using ANOVA with Benjamini and Hochberg with a false discovery rate of 5 % (p-value = 0.05). There was no significance between these genes and Gene Ontology over representation.

Comparison of all family pools via a common wild type reference

The heterozygous, homozygous and wild type pools from both ATM T7271G families were compared to an unrelated wild type control pool for differences in gene expression (Fig. 2C). B-statistics showed that a total of 2068 genes were differentially expressed in at least one pool compared to the unrelated ATM wild type pool. Analysis was carried out to see if these differentially expressed genes are related to ATM genotype. 88 statistically significant differences between the wild type, heterozygotes and

homozygotes pools were found using ANOVA with Bonferoni correction ($p = 0.05$). Clustering of the data reveals some discrete patterns of gene expression, including a group of genes that are over expressed in ATM mutation cells compared to wild type (Fig. 5).

Pairwise comparison of each pool before and after exposure to IR

Carriers of the T7271G ATM mutation have been shown to have an increased sensitivity to ionising radiation (Stankovic et al., 1998). To assess how the T7271G ATM mutation may affect how cells respond to ionising radiation, we compared unirradiated heterozygous carriers, homozygotes and wild type ATM cells from both families to cells irradiated at 3Gy and processed 12 hours post irradiation (Fig. 2D). A total of 1140 genes displayed significant changes in expression level between irradiated and unirradiated cells. B-statistics identified a group of 77 genes that were differentially expressed specifically in the wild type control pools compared to the ATM mutation pools. 2 of these genes (DDX21 and MCM7) that have previously been shown to be differentially expressed 12hrs after 3Gy ionising radiation in ATM wild type lymphoblastoid cells (Jen and Cheung, 2004).
The down regulated genes that were specific to the ATM wild type pools were linked to Gene Ontology and an over representation of genes involved in DNA replication and cell cycle regulation (p-values of 0.000459 and 0.0152 respectively) was found.

Task 3 – Engineer mutant constructs and generate transfections with wild-type and mutant constructs. Do microarray analysis with wild-type and mutant transfections, and cell lines from individuals with different ATM and BRCA1/2 genotypes (months 1-18)

We have constructed the pathogenic *ATM* missense mutation (V2424G; T7271G), which confers a high risk of breast cancer, using cDNA mutagenesis. The mutant and wild-type ATM cDNAs were also tagged with a Green-Fluorescent Protein at their N-terminus. ATM-null cells were transiently transfected with wild-type and mutant ATM cDNA constructs and ATM expression and kinase activity in terms of ability to phosphorylate various targets of ATM *in vitro* and *in vivo* was determined. This data was presented in last year's annual report and the overall summary was that the mutant form of ATM was unable to phosphorylate most of the wild-type ATM targets. A recent study has linked the activation of ATM to its autophosphorylation (Bakkenist and Kastan, 2003). It has been shown that ATM exists as an inactive dimer in cells and exposure to irradiation causes auto phosphorylation on Serine 1981 leading to dimer dissociation and its activation as a kinase. The subsequent monomers then result in rapid and immediate phosphorylation of targets. Therefore, we compared the ability of the wild-type and the mutant ATM protein to auto phosphorylate itself. Data presented in the Fig 1A shows that both the wild-type and the mutant ATM have comparable auto phosphorylation activity *in vitro* and the magnitude of their *in vivo* phosphorylation on Ser1981 after IR was also quite comparable (Fig 1B). Taken together the data suggests that failure to observe phosphorylation of substrates is not caused by lack of auto phosphorylation of mutant ATM protein. This mutant form of ATM appears to separate the auto phosphorylation and substrate phosphorylation functions of ATM. We are at present

investigating the possibility that the mutant ATM protein has a markedly lower affinity for substrates and is thus not able to phosphorylate them both *in vitro* and *in vivo* assays. This pathogenic mutation in *ATM* was also constructed in the EBV-based episomal vector, pMEP4, under the control of metallothionein II-inducible promoter and the control and AT cell line stably expressing the mutant form of ATM were selected. We were unable to perform biochemical and phenotypic aspects of ATM function using this construct as the concentration of the cadmium chloride required to induce ATM expression caused a significant amount of DNA damage resulting in activation of ATM signalling pathways independent of ATM. An example of this is shown in Fig 6, in which cadmium chloride is shown to cause significant stabilization of p53. Due to this problem, the mutant and wild-type ATM cDNA have been constructed in the constitutive EBV-based episomal vector, pREP4 and the control and AT cell line stably expressing the mutant form of ATM have been selected and are being analysed at present.

Task 4 – Extend the analysis of ATM kinase activity to all available family members based on results with the index cases (months 13-18).

LCLs from 7271 and IVS106T-G families have been established and all the family members were genotyped for mutations and LCLs of all mutation carriers and some of the non-carriers (normals) in the family were tested for phosphorylation of p53 on Ser15. The data obtained was inconsistent as 1 of the 9 cell lines tested from non-carriers had reduced activity whereas half of the carriers tested had normal ATM activity. We are currently evaluating various antibodies against other ATM targets including CHK1, CHK2 and BRCA1 to find a target, which consistently gives reproducible results.

Task 5 – Start mutation analysis of the ATM gene in families with compromised ATM kinase activity (months 15-24).

DNA from the 6 index cases found to have aberrant ATM kinase activity have been screened for mutation in ATM by D-HPLC to determine whether the lack of activity is due to mutations in *ATM*. We found one 7271T-G mutation, one IVS 10-6T-G and no additional mutations were found in the remainder 4 samples but some common polymorphisms as well as intronic changes were found, which are being further evaluated initially by their frequency in controls,

Key Research Accomplishments

1 252 LCLs have been established from index cases from non-BRCA1/2 breast cancer families.
2 All LCLs have been analysed for ATM expression and kinase activity using anti-phospho Ser15 antibody.
3 12/252 (4.7%) LCLs showed markedly reduced ATM kinase activity, despite normal level of expression of ATM protein.
4 Microarray profiling of 2 ATM T7271G families containing heterozygous, homozygous and wild type members.

5 Data extraction, image analysis and data management of microarrays.
6 Analysis of gene changes occurring between different ATM genotypes.
7 Comparison of profiles for T7271G ATM heterozygotes with or without breast cancer.
4 8 Analysis of whether ATM genotype may affect how cells respond to ionising radiation.

Reportable outcomes

- Presented at the Breast Cancer Linkage Consortium, Madrid, June 2003.
- Presented at the 3rd Australian Microarray Conference, Couran Cove, July 23rd-26th 2003
- Presented at Ataxia-telangiectasia and ATM International workshop, Fraser Island, September 2003.
- Presented at the Lorne Cancer Conference, February 2004
- 252 lymphoblastoid cell lines have been established from index cases of multiple case-breast cancer families with no pathogenic mutations in BRCA1/BRCA2. These are available to other researchers through kConFab (http//:www.kconfab.org).
- An Excel database has been established to record receipt of LCLs, and the results of assays of ATM function.

Conclusions

12/252 (4.7%) lymphoblastoid cell lines from index cases of multiple-case breast cancer families have been identified with markedly reduced ATM kinase activity suggesting that these cell lines might contain pathogenic mutations in ATM. Pathogenic mutations in *ATM* were found in 2 of the 6 cell lines tested. involved.

Microarrays have been performed to determine differences in gene expression between heterozygotes (with or without breast cancer) or homozygotes for the T7271G mutation against each other and wild type control pools. The response of these different cells to ionising radiation has also been investigated.
These data have suggested candidate genes that are altered in their expression in T7271G *ATM* mutation carriers and how these mutations may affect the cells response to ionising radiation. However it is difficult to determine what genes may be involved in disease state and the genesis of ATM-related breast tumours. The data must now be verified and can then be challenged with other carriers of ATM T7271G to see if it can be used to develop an alternate method for high-throughput screening of ATM mutations.

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Figure legends

Fig.1 Functional analysis of V2424G (base T7271G) mutant ATM protein. HEK-293T cells were transiently transfected with a GFP vector alone, GFP-wt ATM and GFP-mutant ATM. Whole cell extracts were prepared and immunoprecipitated with anti-GFP antibody (Panel A). In vitro kinase assays were performed on immunoprecipitated ATM with GST-p53 (aa1-40) as a substrate or without substrate (auto phosphorylation) and

γ^{32}P ATP. Samples were analysed by SDS-PAGE, followed by immunoblotting with anti-GFP antibody to determine protein expression and autoradiography to measure incorporation of phosphate groups into p53 and ATM. *In vivo* autophosphorylation activity of the constructs transfected in ATM-deficient cell line AT5BIVA was assessed by immunoblotting with anti-phosphoserine-15 antibody (Panel B). The cell extracts were prepared with (+) or without (-) prior exposure of cells to 6 Gy ionizing radiation (IR).

Fig.2 Microarray experimental design to analyse changes between the pooled ATM members. To answer whether microarray analysis can be used to identify families carrying dominant negative mutations in ATM the experiment was divided into 4 sections:
A. A comparison of different ATM genotypes (heterozygous m/+ and homozygous m/m) via an ATM wild type pool was carried out
B. To detect genes that may be involved in the genesis of ATM-related breast tumours a comparison of profiles for heterozygotes with cancer Vs those with no breast cancer relative to wild type was performed
C. An unrelated wild type control pool was used as a common reference to compare all pools
D. To assess how ATM mutations may affect the cells response to ionising radiation a pair-wise comparison of each pool before and after exposure to ionising radiation was performed
Arrows between the pooled samples represent one microarray experiment, for each comparison microarrays were carried out triplicate with one dye swap. Numbers in brackets indicate how many members are in each pool.

Fig. 3 Box plots displaying the transformation steps carried out within and between all the microarrays. The expression data for all genes on each array is represented by one box plot, which is plotted against the $\log_2$ ratio on the y-axis. The box stretches from the lower hinge (defined as the 25th percentile) to the upper hinge (the 75th percentile) and therefore contains the middle half of the scores in the distribution. The median is shown as a line across the box. Lines are drawn from the box to the inner fence. Gene outliers between the inner and outer fences are indicated by an "o".
The initial normalization step is a print tip loess normalisation that was carried out within each array. This data was then subject to scaling between arrays (Fig. 3B).

Fig. 4 Volcano plot displaying the results of B-statistics. To test the significance of the data BASE was used to calculate the B-statistics of all genes between each of the different pools. The probability (B-score) is plotted against the Log fold change for each gene, thus significance and magnitude of each gene between conditions can be visualised. In this sample the heterozygous pool (family 1) is being compared against the unrelated wild type control pool. Gene names and identifiers of significant genes are shown at the top of the plot.

Fig. 5 Cluster of genes with statistically significant differences between wild type control pools, heterozygote and homozygote members of the 2 families. ANOVA with Bonferoni

correction ($p = 0.05$) was carried out on all genes. The 88 genes that were found to have statistically significant differences between ATM genotype were clustered. A region in the cluster containing genes that are over expressed in the heterozygous and homozygous pools compared to wild type control pools is shown in greater detail and includes gene identifiers and Genbank accession numbers.

Fig. 6 Stable transfection of control (C3ABR) and AT (AT3ABR) cell line with mutant form of ATM (V2424G). C3ABR and AT3ABR cell lines were transfected with Flag-tagged V2424G mutant ATM cDNA cloned in pMep4 vector, and stable cell lines were established by selection in hygromycin. Expression of mutant ATM protein was induced with cadmium chloride for 6 h before irradiation (6 Gy IR) and incubation for 30 min. ATM expression was determined by immunoprecipitation with anti-flag followed by immunoblotting with same antibody and p53 phosphorylation was determined by blotting with anti-phospho Ser15 p53.

Fig 1A

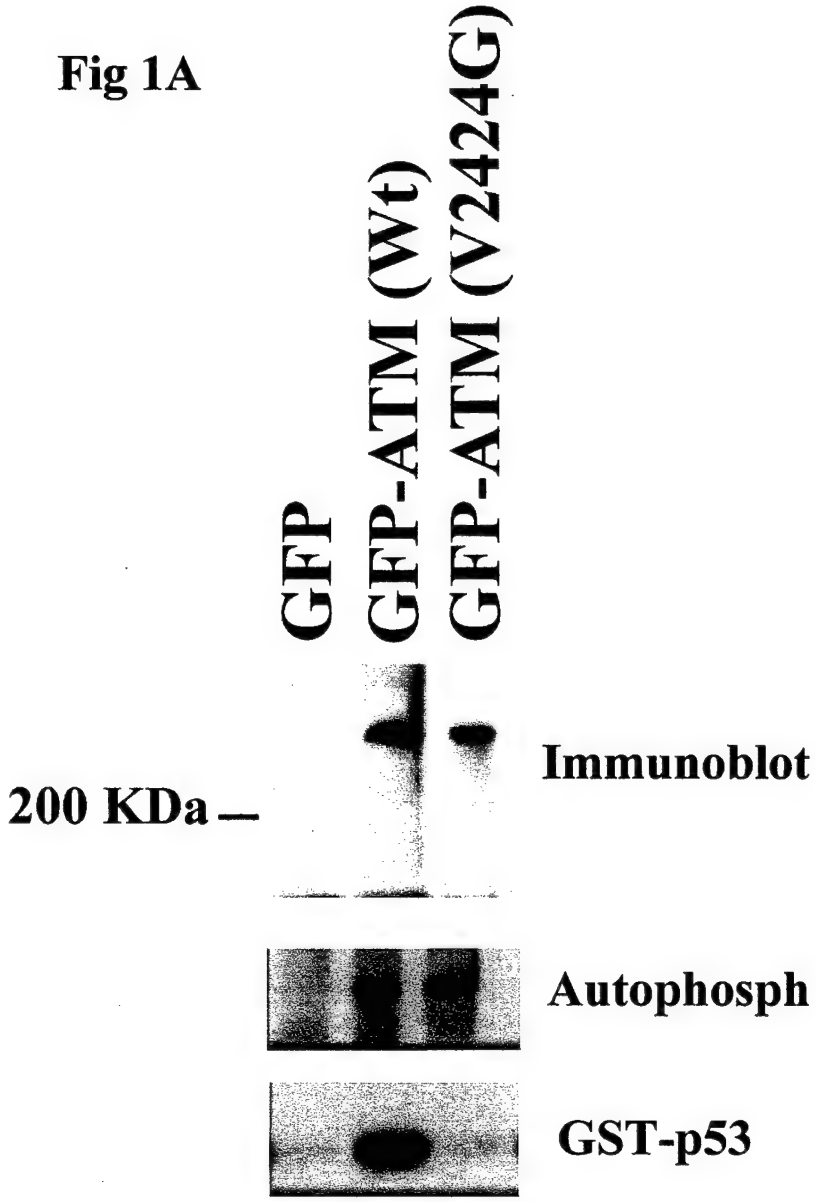
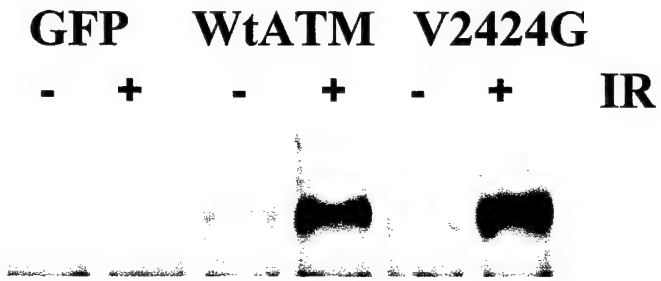
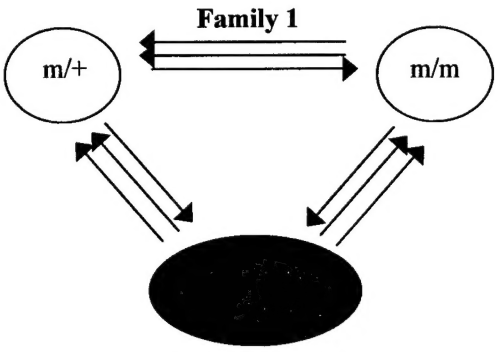


Fig 1B



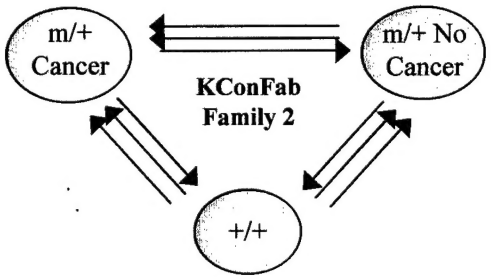
WB: anti Ser-1981

Fig. 2 Experimental Design



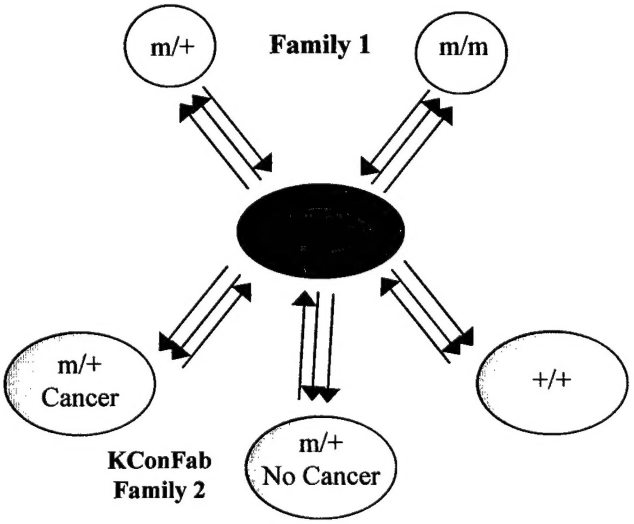
m/+ = heterozygote pool (2)
m/m = homozygote pool (3)
+/+ = wild type pool (6)

A: Comparison of all three genotypes in Family1



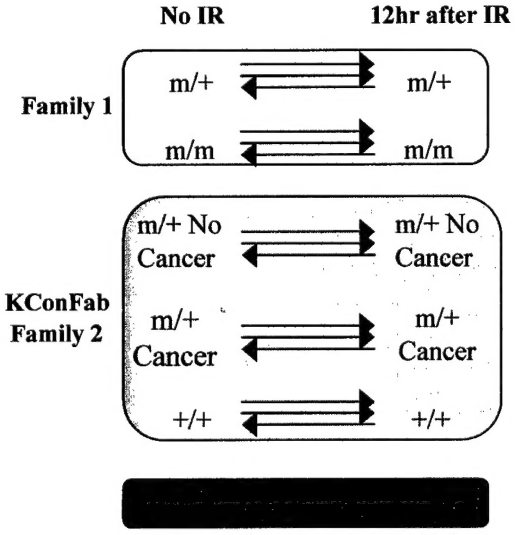
m/+ No Cancer = heterozygote pool (3)
m/+ Caner = heterozygote pool with breast cancer (3)
+/+ = wild type pool (6)

B: Comparison of profiles for heterozygotes with disease Vs not, relative to +/+ in Family2



m/+ = heterozygote pool
m/m = homozygote pool
+/+ = wild type pool

C: Comparison of all pools via a common reference

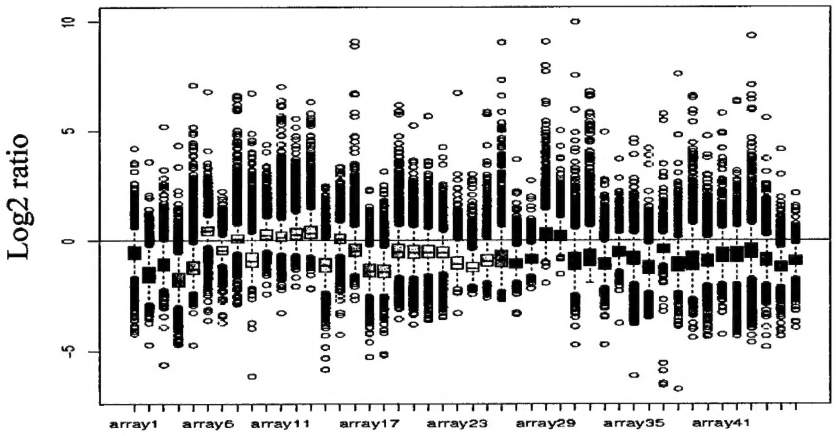


m/+ = heterozygote pool
m/m = homozygote pool
+/+ = wild type pool

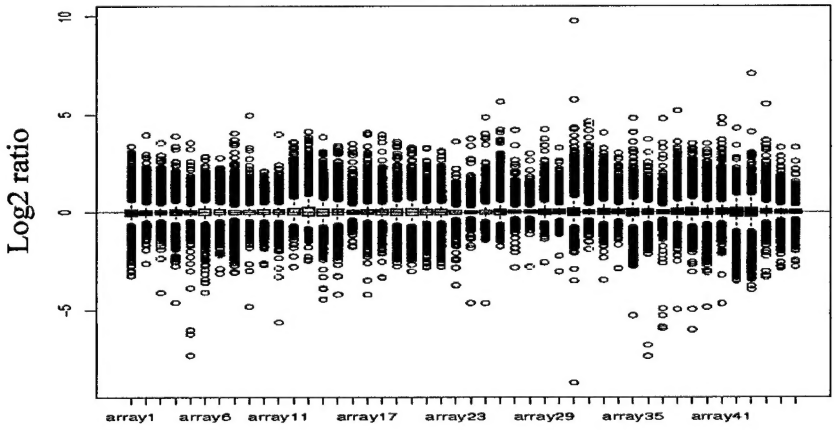
D: Pair-wise comparisons of each pool before and after exposure to IR

Fig. 3 Data transformation:

A. Raw data



B. After Loess normalisation



C. After scaling

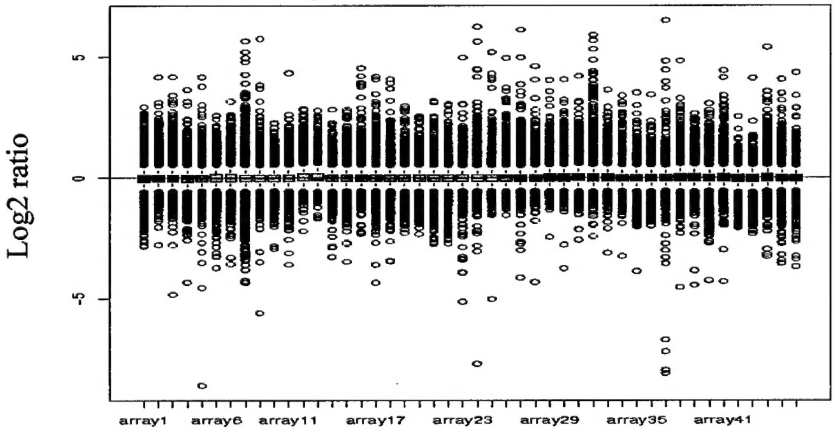


Fig. 4 Plotting significance of differential expression using B-statistics

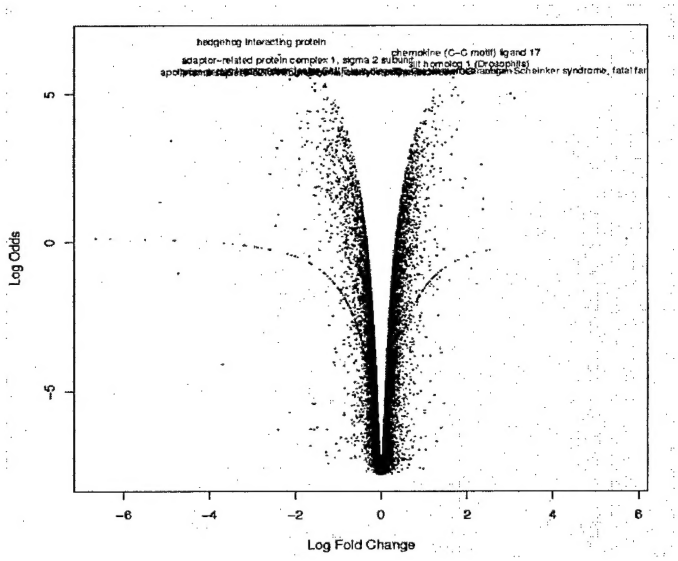


Fig. 5 Hierarchical Clustering of Genes Associated with ATM Genetic Characteristic

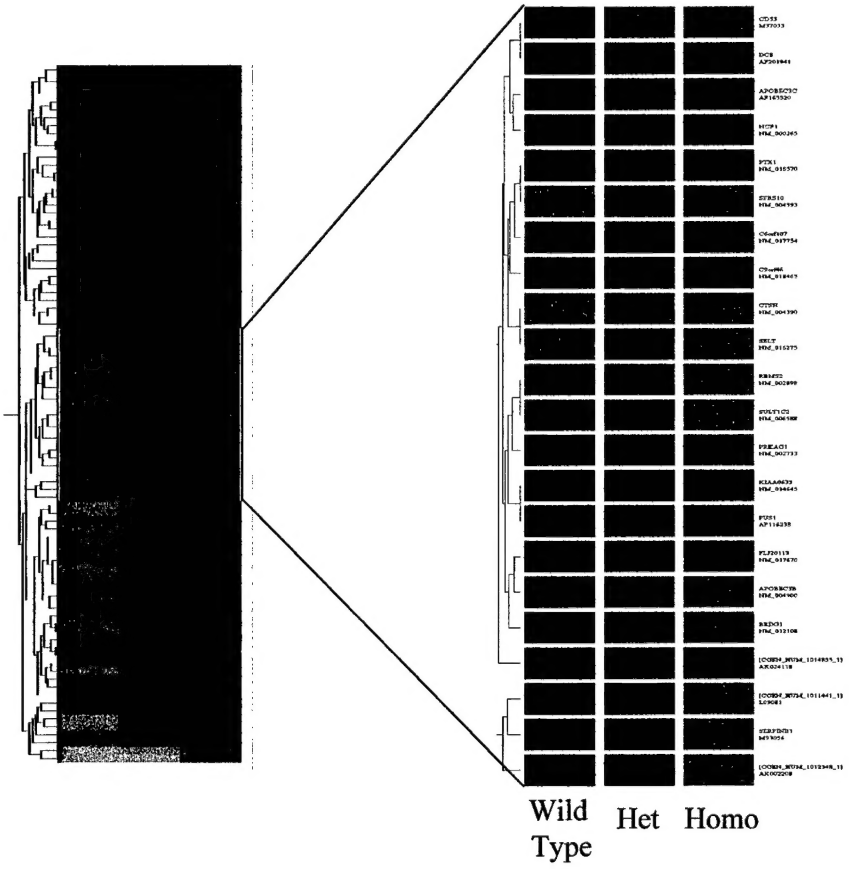


Fig.6